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Journal of Molecular Catalysis B: Enzymatic 11 (2001) 825–834

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Characterisation of a novel support for biocatalysis in supercritical carbon dioxide

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Abstract

The work presents a characterisation study of Accurel EP100 (polypropylene based hydrophobic granules) as support material for lipase (Lypozyme 10,000 l, from native *Rhizomucor miehei)* operating as biocatalyst in supercritical CO_2 as solvent. The study involved assay of biocatalytic activity and operational stability as functions of system pressure and temperature. Furthermore, the presence of diffusion limitations was tested, by varying the bed diameter and support particle size. In addition, SEM and Gas Absorption were employed to test the mechanical stability. Results were compared with the commercially available biocatalyst Lipozyme™ IM60.

Pressure did not have a significant effect on the activity or the stability, while temperature had a positive effect on the activity and negative effect on the stability. As expected, an 'optimum' value of system water content gave maximum catalytic activity for each biocatalyst. External- and internal-diffusion limitations were both found negligible. The mechanical stability analysis demonstrated little (if any) effect of supercritical carbon dioxide ($scCO_2$) on the structural integrity of Accurel EP100, although subtle increases in pore volume and surface area were observed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Biocatalysis; Supercritical carbon dioxide; *Rhizomucor miehei*

1. Introduction

Lipases are of particular interest to the food and pharmaceutical industries due to their stereo- and regio-specificity, that facilitates the production of specialist compounds that are unobtainable by the known chemical route. The enantio-selectivity of lipases is useful for resolving optically active mixtures such as ibuprofen and derivatives [1]. Also, they operate under mild conditions, the factor that preserves the hygiene and quality of foods and pharmaceutical products.

There is much evidence in literature about the suitability of supercritical fluids as media for enzymatic reactions [2–8]. Supercritical carbon dioxide ($scCO_2$) offers excellent characteristics as solvent due to its high (liquid-like) density and solvating power, low viscosity, high diffusivity and low surface tension, which reduces substrate-diffusion limitations. Most importantly, it is readily removable by post-reactional de-pressurisation. This offers great advantage especially for product purity at reduced

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PII: S1381-1177(00)00195-8

cost, avoiding the expense and complexity of separation processes. In most industrial processes continuous systems would be more economically favourable, and therefore require attention [9–11].

Numerous reports were presented on the excellent activity and stability of the commercially available immobilised lipase, Lipozyme™ IM60 in $scCO_2$. It consists of lipase from *Rhizomucor miehei* cloned into *Aspergillus oryzae*, immobilised on Duolite, the anion exchange resin. Yet, a limited number of other supports were shown to perform well in high-pressure environment. This includes derivatised glass beads, celite, Hyflo Supercel and PMMA (polymethyl-methacrylate). However, the use of Accurel EP100 has only recently been reported in $scCO_2$ [12]. It is inexpensive and was shown to be very effective in a variety of environment [13].

This work presents a characterisation study of (Lipozyme 10,000 l/Accurel EP100) biocatalyst employed for reactions in $scCO_2$, in comparison with the commercially available Lipozyme™ IM60.

2. Experimental section

2.1. Materials

2.1.1. Support

Accurel EP100 (from Akzo Nobel, Obernburg, Germany) was the selected support material. It is polypropylene-based hydrophobic granular support. Its properties are given in Table 1.

2.1.2. Lipases

Lipozyme™ IM60 (lipase from *R. miehei* cloned into *A. oryzae* and immobilised on the anionic resin, Duolite), and Lipozyme 10,000 l (free lipase from *R. miehei*) were supplied by Novo Industri (Bagsvaerd, Denmark).

Table 1
Physical properties of Accurel EP100

Property	Description
Support form	Granules
BET surface area (m^2/g)	42.95
Particle size distribution (μm)	63–125, 400–1000
Particle density	902 kg/m^3 (or 0.902 g/cm^3)
Particle voidage	0.75

2.1.3. Substrates and reagents

Oleic acid (92% w/w), oleic acid standard (99–100%) and ethyl oleate standard (99–100%) were obtained from Sigma Chemical Co. (St. Louis, MO) and ethanol (99–100%) was purchased from BDH (Poole, England). For economic reasons a low purity oleic acid has been used for all continuous reactions. BOC Ltd. (Guilford, Surrey) supplied carbon dioxide (99.5% w/w) of industrial grade. Methanol of Certifi HPLC grade and HPLC grade acetic (glacial) acid were obtained from fisher Scientific (Pittsburgh, PA) for HPLC analysis.

2.2. Methods

2.2.1. Lipase immobilisation

The immobilisation procedure is briefly stated below, as more details can be found in previous work [14].

In preparation for immobilisation, the support was pre-wet with 50 ml ethanol for 30 min and pre-washed with 50% ethanol–water, to exclude the air within the support particles. The mixture was then washed with 50 ml de-ionised water, decanted then washed with 100 ml fresh de-ionised water and filtered ready for immobilisation.

Upon immobilisation, 1 g of prepared support was brought into contact with 50 ml of lipase solution (of predetermined initial concentration[1]) in a sealed vessel, and placed in an orbital shaker at 250 rpm and 30°C (optimum temperature for all the lipases used in this study) for 48 h. Then the lipase laden support particles were filtered from solution, dried and stored at 4°C, ready for the assay of immobilised lipase activity. The amount of lipase adsorbed (lipase loading) was defined in terms of lipase unit (LU) per unit

[1] A series of lipase solutions of consecutively increasing concentrations was prepared, immobilised and the activity of each immobilised lipase was measured by esterification of oleic acid and ethanol. The initial lipase concentration that corresponded to the highest activity was selected for the present study.

weight of support and was determined by subtracting the residual free lipase activity (after immobilisation) from the original free lipase activity (before immobilisation).

2.2.2. Equipment and analysis

Fig. 1 describes the experimental unit employed in the present work, which is a modified Milton Roy (LDC Division, Rivera Beach, FL) Sample Preparation Accessory (SPA™) Unit. Substrates namely oleic acid, ethanol and water were premixed and continuously injected into the solvent stream at the mixer (M201), by a single piston HPLC pump (P102). Substrate and solvent flow rates were based upon physical data of CO_2 provided by Angus et al. [15]. Substrate concentrations were validated using HPLC analysis. Water concentration was based upon the amount entering the fluid phase.

The packed-bed reactor was a 4.6 mm × 3.7 mm empty HPLC column. After the reaction, a novel six-way pneumatic valve (V202) allowed the automatic withdrawal of samples from the process stream and onto an HPLC column for analysis. Such *on-line* mechanism allowed almost instantaneous reports on the progress of the reaction. Four samples, 25 min apart, are taken for each residence time and analysed

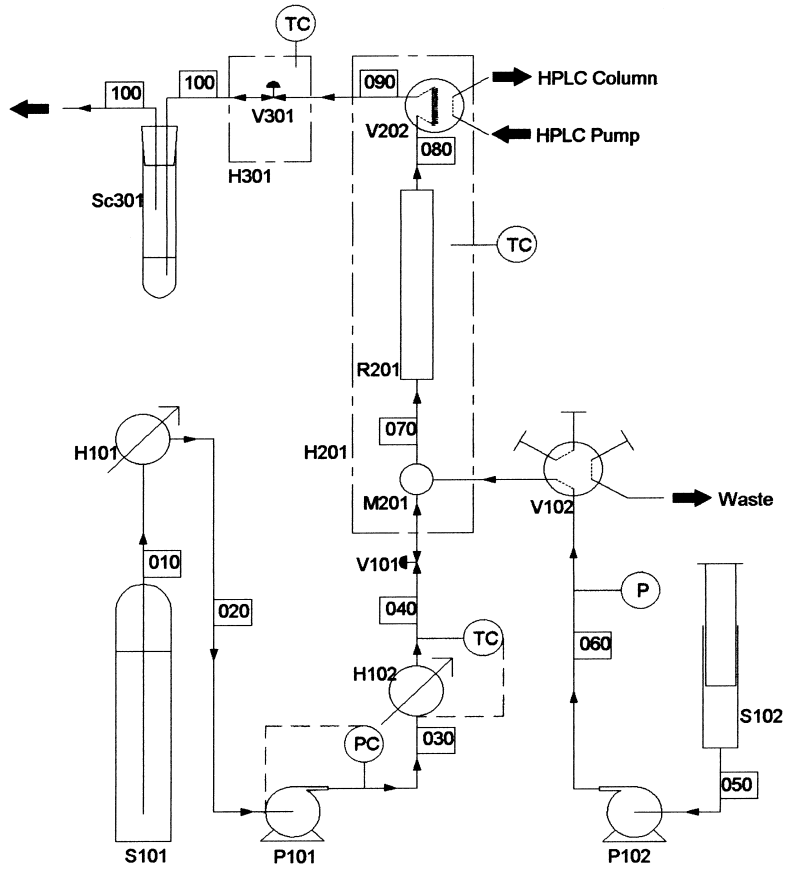

Fig. 1. Modified sample preparation accessory unit: S101, liquid CO_2 cylinder; H101, chiller; P101, high pressure dual piston pump; H102, thermostatically controlled heater; S102, substrate storage; P102, Gilson 305 HPLC (France) pump; V101, isolation valve; V102, six-way valve; M201, static mixer; H201, thermostatically controlled heated air bath; R201, packed bed reactor; V202, six-way pneumatic sampling valve; V301, Rheodyne back pressure regulator; H301, thermostatically controlled water bath; Sc301, waste trap. TC, temperature control; PC, pressure control; P, pressure.

Table 2
The structural properties of Accurel EP100

Average pore diameter (μm)	0.1975
BET surface area (m^2/g)	42.95
Pore volume (PV) parameters	
Total pore volume (cm^3/g)	3.056
Most frequent diameter (μm)	7.350
Diameter at 50% pore volume (μm)	2.17
Pore surface area (SA) parameters	
Total pore surface area (m^2/g)	62.4
Most frequent diameter (μm)	0.0205
Diameter at 50% SA (μm)	0.0281

by HPLC. Detection was carried out at 210 nm. All reactions were carried out at 40°C and 13 MPa. Equilibrium is achieved 30 min after start-up.

3. Results and discussion

As mentioned earlier, Accurel EP100 is a polypropylene-based hydrophobic granular material that has shown high affinity towards lipases. However, prior to investigating the activity of the biocatalyst in $scCO_2$, it is important to test the mechanical stability of Accurel.

3.1. Mechanical stability

Table 2 gives the structural properties of Accurel EP100.

Clearly, the BET surface area based on monolayer nitrogen absorption is considerably smaller than the total pore surface area, which shows that most of the surface area, is concentrated in the micropores. Table 2 shows that the most frequent diameter (in micropores) is larger than that of the lipase ($\sim 0.0075\,\mu m$) by several orders of magnitudes [16]. This would make lipase adsorption on Accurel, diffusion controlled rather than geometry restricted. It is generally accepted that the entry pores have to be approximately 4–5 times the molecular diameter of the enzyme for unrestricted access to occur [17]. For the *R. miehei* lipase when the pore diameter drops below 0.025 μm, access to the internal structure becomes difficult.

Plates 1–3 show SEM images of Lipozyme IM60 and Accurel EP100 before and after exposure to $scCO_2$. In Plate 1 Accurel EP100 demonstrated a heterogeneous surface with a network of macropores. Within such matrix exists the micropore network. A sample of Accurel was placed in a glass vial in a stainless steel vessel fitted with a sapphire window and exposed to pressures up to 18 MPa and

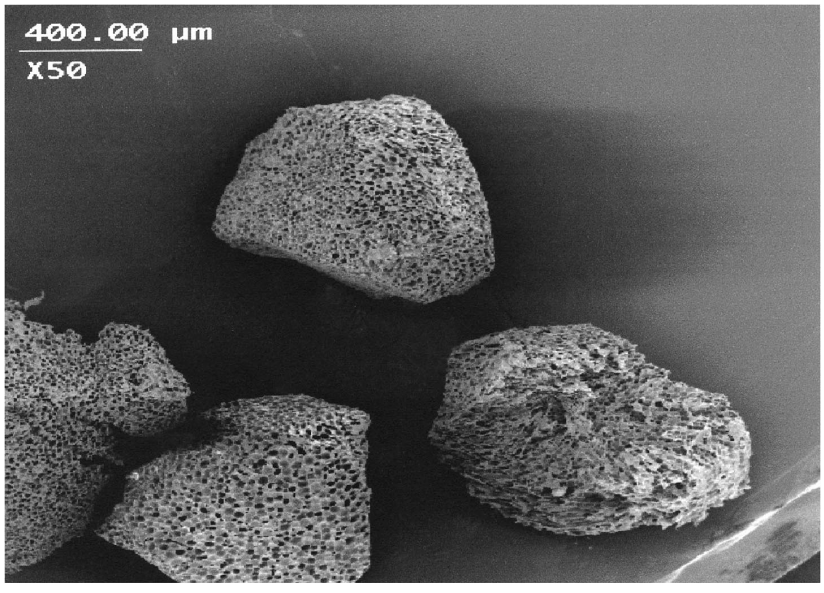

Plate 1. SEM image for Accurel EP100.

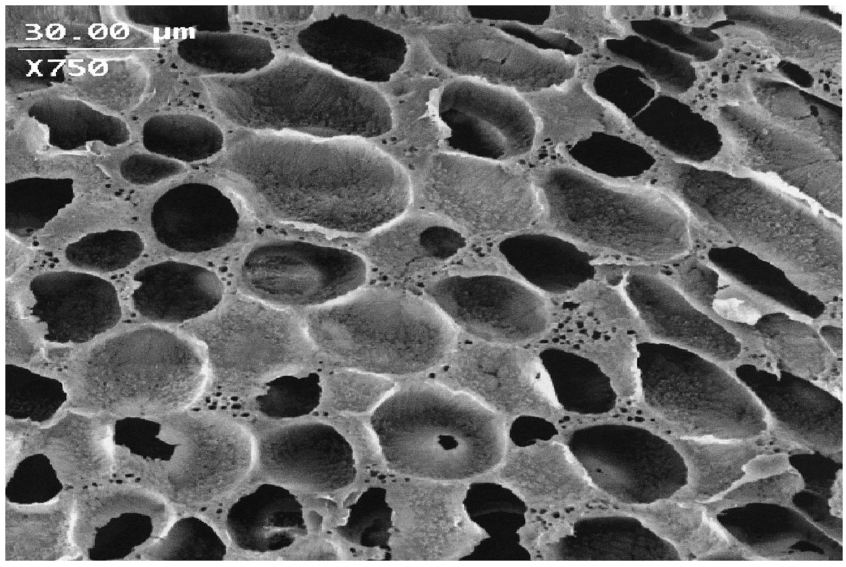

Plate 2. SEM image of Accurel EP100 (before exposure to $scCO_2$).

temperatures up to 60°C over 4 h. The sample was visually analysed during such exposure to determine the extent of any possible temporary changes in the physical structure of the polymer, e.g. swelling. However, at all operating conditions, the polymer appeared to visually retain its size and structure.

To determine if more subtle changes occurred in the structure, Gas Absorption was carried out on the

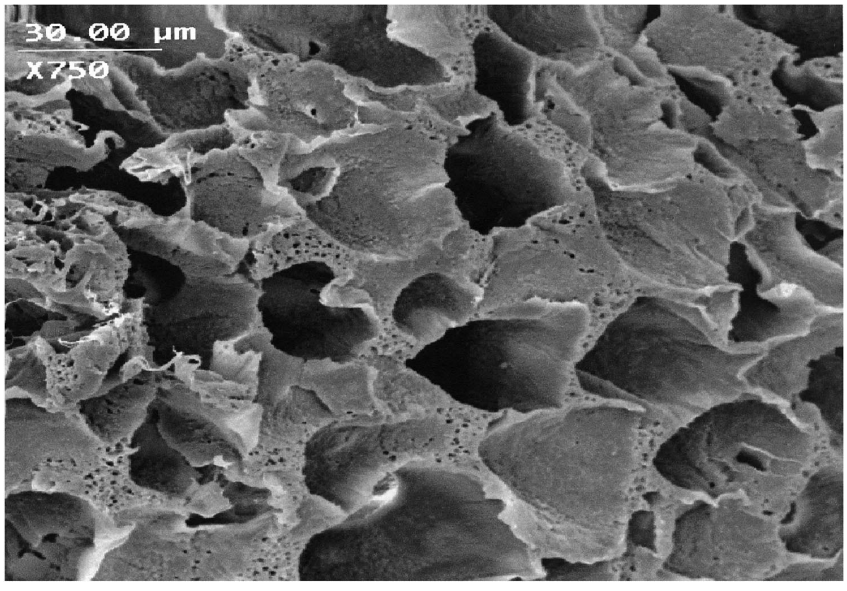

Plate 3. Accurel EP100 after exposure to $scCO_2$ (6 h, 13 MPa, 40°C).

Table 3
Results of nitrogen adsorption for the effect of $scCO_2$ on Accurel EP100 (13 MPa, 40°C, 6 h)

	Surface area (m^2/g)	Pore volume (cm^3/g)
Before	42.95	7.35
After	47.8	10.97

samples before and after exposure to $scCO_2$. Interestingly, a significant increase in both the surface area and the pore volume was observed and the results are shown in Table 3.

This might indicate the loss of some support material as suggested by Shieh et al. [18].

3.2. Selection of the optimum water activity

Generally in lipase catalysed reactions, water acts as enzyme hydrator. Proper hydration state would be necessary for the enzyme to reach its active conformation. Water solubility in $scCO_2$ determines water distribution between the biocatalyst and the solvent, which is obtained from water adsorption isotherm [10]. Therefore, the total amount of water added to the system is the hydration water plus the amount adsorbed on the support.

In the present work experiments were conducted to find the optimum water content that would give maximum activity for each biocatalyst, and the results are shown in Fig. 2. The initial lipase activity (corresponding to $< 15\%$ conversion) was represented by the rate of esterification of oleic acid and ethanol to synthesise ethyloleate (number of μmol produced) in unit time using a unit mass of biocatalyst. The amount of water to be injected with the solvent into the system was back calculated from the adsorption isotherm using the required amount of adsorbed water. As expected a bell-shaped curve was obtained, indicating that a certain amount of water was required to open the 'lid' for most active lipase conformation. Any excess water in would attack the hydrophilic barrier, and lead to loss in enzyme activity, and in some cases, enzyme de-naturation. This phenomenon was encountered in previous literature [7,10,11,19,20]. Fig. 2 also shows that at 40°C and 13 MPa, the optimum water contents were 0.95 and

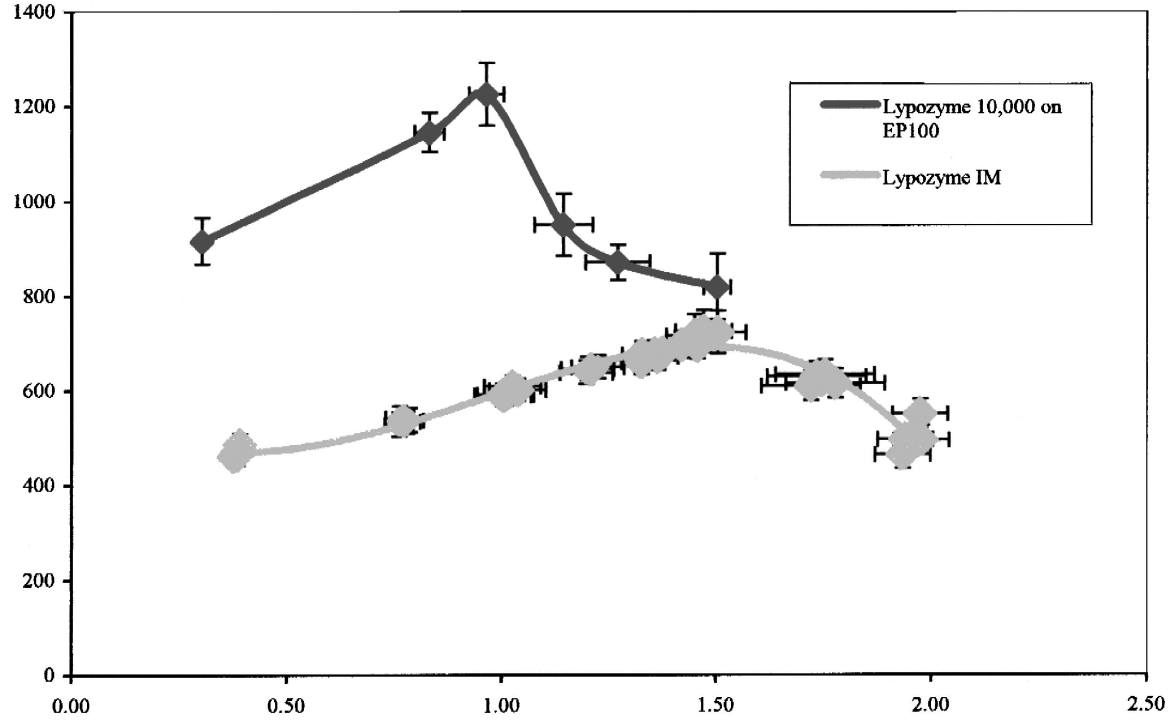

Fig. 2. Effect of water content on the initial enzyme activity for the three biocatalysts under investigation.

1.5 g/l for (Lipozyme 10,000 l/Accurel EP100) and Lipozyme™ IM60, respectively. Furthermore, the former showed significantly higher activity range than the latter. The difference in optimum water content in the two systems was attributed to the difference in surface topographies of the supports, resulting in different adsorption energies and different water distribution.

3.3. The effects of pressure and temperature

Fig. 3 shows the effect of pressure and temperature on the activity of (Lipozyme 10,000 l/Accurel EP100). Results suggest that increasing the pressure from 13 to 18 MPa did not significantly effect the lipase activity. Generally, as pressure increased the density and viscosity would increase the factor that might enhance diffusion limitations on the one hand. On the other hand the increased substrate solubility (with pressure) would counteract the above effect, resulting in negligible net effect on the lipase activity.

Fig. 3 also shows that the lipase activity increased when temperature increased from 40 to 60°C, and hence the rate constant increased too. Also, the substrate viscosity and density decreased, reducing the diffusion limitations and further enhancing the reaction rate and hence the lipase activity. In general, temperature increase could also cause de-naturation of enzyme stopping the reaction altogether. However, in the present system, the experimental temperature range did not cover that possibility.

Figs. 4 and 5 show the effect of pressure and temperature, respectively, on the operational stability of the biocatalyst. Pressure changes had insignificant effect on the stability, while temperature had a prominent effect. At 60°C the enzyme lost 30% of its activity over 8 h, compared to 15% loss at 40°C. This confirms the negative effect of higher temperature on the enzyme structure leading to de-naturation. Such loss of enzyme stability with temperature was encountered elsewhere [21].

3.4. Mass transfer effects and diffusion limitations

The presence of external mass transfer and intraparticle diffusion limitations in the present system was also investigated.

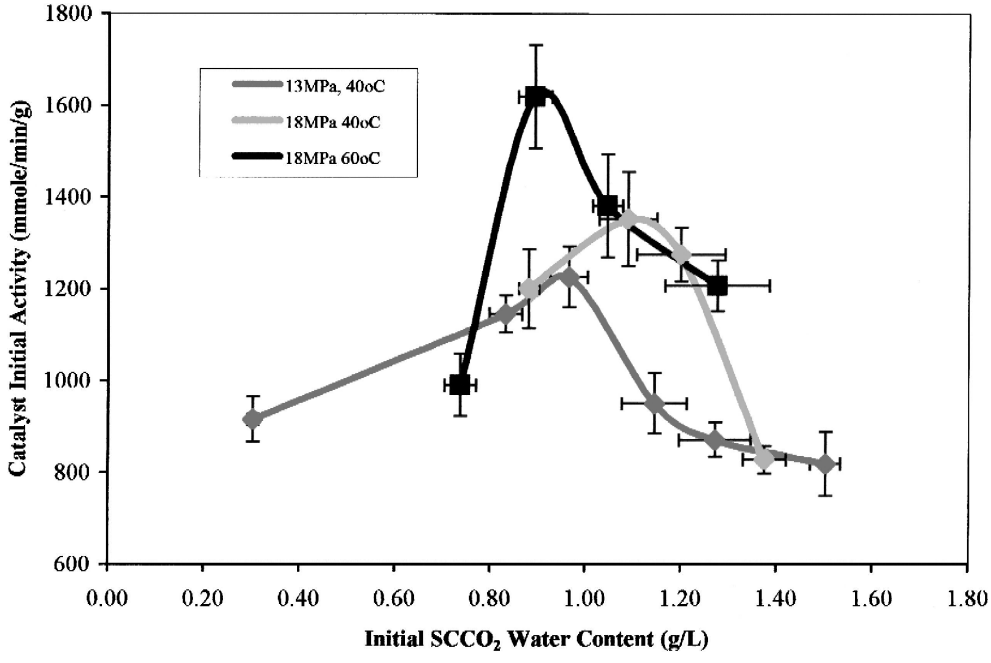

Fig. 3. Effects of temperature and pressure on the catalytic activity of (Lypozyme 10,000 l/Accurel EP100) system.

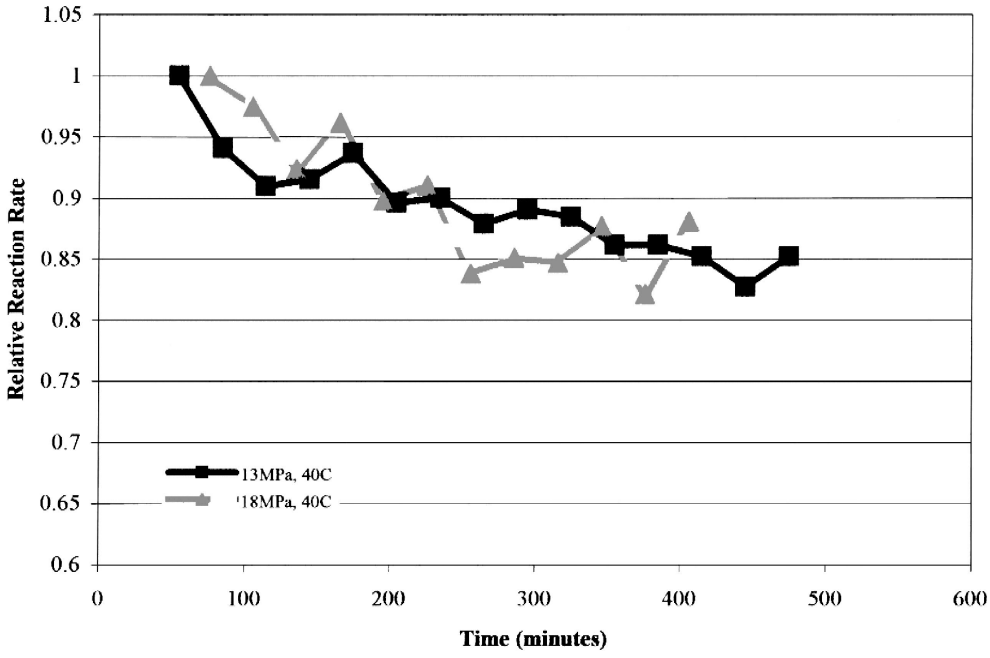

Fig. 4. The effect of pressure on biocatalyst stability for (Lipozyme 10,000l/Accurel EP100) system.

3.4.1. External mass transfer

Generally, external (film) transfer describes transport from the substrate bulk across the boundary layer to the outer surface of the biocatalyst. Reducing the boundary layer through increased fluid superficial velocity would therefore eliminate film limita-

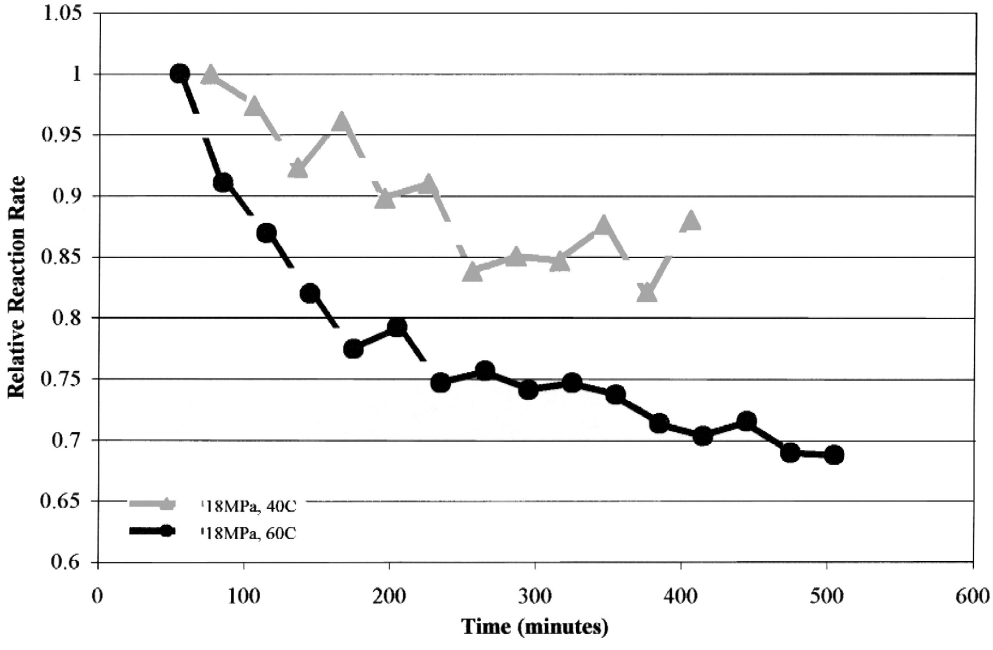

Fig. 5. The effect of temperature on biocatalyst stability for (Lipozyme 10,000l/Accurel EP100) system.

tions. In the present work, the effect of film transfer was detected by changing the superficial velocity, using two different bed diameters namely 4.6 and 10 mm. Results show very small difference in the conversion rate indicating that film transfer limitations were negligible. This would be expected, given the excellent transport properties of $scCO_2$.

3.4.2. Intraparticle diffusion

Intraparticle diffusion limitations are function of the structural properties of the solid support particles namely the pore size, pore volume, surface energy distribution and tortuosity. Therefore, the presence of limitations could be detected by varying the particle size distribution of the support particles. In the present work, two size ranges were used namely 200–400 and 400–1000 μm. Results suggested that intraparticle diffusion was unlikely to have significant effect on this system. A similar approach was applied to glass beads [22–24] in $scCO_2$; all obtained similar results. For Lipozyme™ IM60 system, particle ranges of 63–125 and 400–800 μm were tested. Interestingly, esterification rates in $scCO_2$ (13 MPa, 40°C) were found to be 130% higher for the smaller size range, indicating significant diffusion limitations in the higher size range. For the same lipase loading, less of the lipase laden surface area would be accessible for the substrates, the matter that would make diffusion to the active sites more significant. Similar results were observed in literature for the same biocatalyst [25,26].

3.5. Operational stability

Fig. 6 shows the operational stability over 20 h in $scCO_2$ conditions. In general, all biocatalysts demonstrated good stability keeping over 90% of the original activity throughout the operation time. Yet, Lipozyme™ IM60 was reported to exhibit excellent stability in a variety of environments including $scCO_2$ [10]. However, this work shows that the operational stability of Accurel EP100 systems depended on the degree of conversion of oleic acid, indicating a possible substrate destabilisation effect. It was particularly evident in (Lipozyme 10,000 l/Accurel EP100) system when, upon operating at $< 20\%$ conversion, it lost nearly 15% of its stability within the first 8 h of operation.

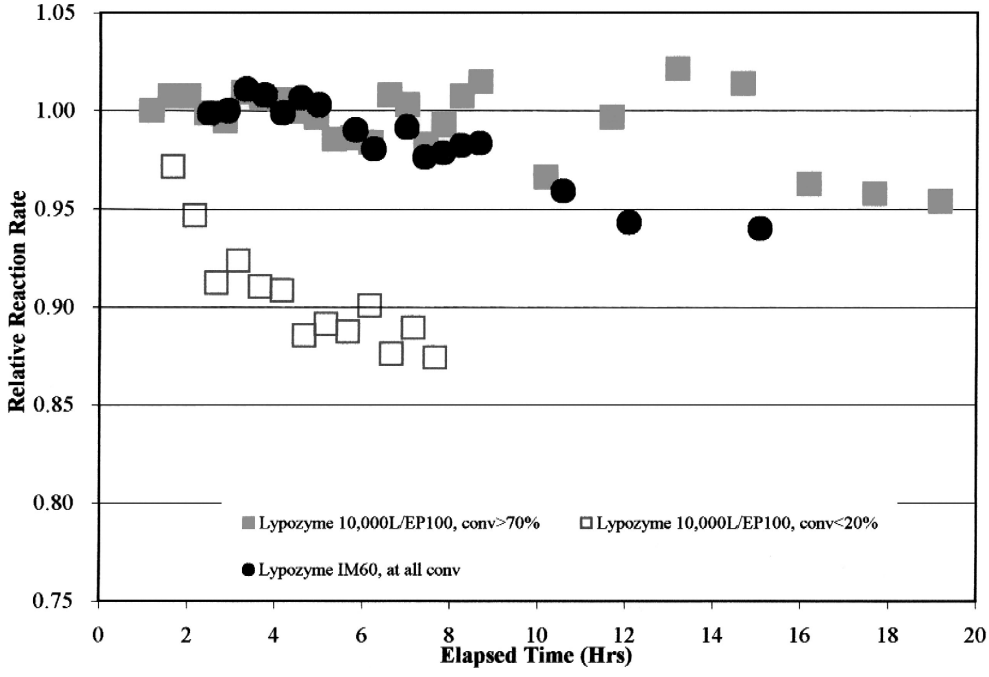

Fig. 6. Effect of conversion on the operational stability of the biocatalysts under investigation.

4. Conclusions

In conclusion, (Lipozyme 10,000 l/Accurel EP100) exhibited competent properties as biocatalyst in supercritical media.

- It exhibited negligible external and internal diffusion limitations.
- It showed good operational- and mechanical stability in $scCO_2$. Certainly, no major physical or structural changes were observed up to 18 MPa and 60°C, though changes in pore volume and surface area were observed and attributed to a possible loss of support. It is, therefore, recommended that the support be subjected to $scCO_2$ prior to immobilisation and usage.

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